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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
The epidermal growth factor receptor (EGFR) is an important mediator of breast cancer tumorigenesis and metastasis. While much is known about EGFR signal transduction related to its tyrosine kinase activity, less is known about the protein tyrosine phosphatases (PTPs) which must be present to modulate the cellular effects of the EGFR by dephosphorylating the receptor and its substrates. Evidence derived from several approaches suggests that the transmembrane PTP LAR may be involved in EGFR signaling in mammary gland development and tumorigenesis. The *hypothesis to be tested in this proposal is that LAR plays an important role in EGFR-dependent mammary gland development and tumorigenesis through negative modulation of EGFR signal transduction.* Work in the second year has supported this hypothesis. LAR expression is also shown to be regulated by cell density, with concentrations increasing markedly as cell density increases. Functional E-cadherin complexes are necessary for this effect. Cell contact inhibition of EGF-dependent proliferation in MCF10A mammary tumor cells is shown to be mediated at the level of Akt activation. E-cadherin complexes are presumed to be the mediators of this cell contact inhibition. Thus, it is intriguing to speculate that the density dependent increase in LAR by E-cadherin complexes and the suppression EGF signaling by E-cadherin complexes are linked. A remaining experimental question is whether LAR levels increase with cell density in order to suppress EGFR signaling.

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INTRODUCTION

The epidermal growth factor receptor (EGFR) and the related protein erbB2 have been implicated as important mediators of breast cancer tumorigenesis and metastasis. While much is known about EGFR signal transduction related to its tyrosine kinase activity, less is known about the protein tyrosine phosphatases (PTPs) which must be present to modulate the cellular effects of the EGFR by dephosphorylating the receptor and its substrates. Evidence derived from several approaches suggests that the transmembrane PTP LAR may be involved in EGFR signaling in mammary gland development and tumorigenesis. Two sets of data are particularly important. First, the LAR knockout mouse has been shown to have a defect in terminal mammary gland development. Second, we have shown that suppression of cellular LAR by 60% using an antisense expression vector results in a 3-4 fold elevation of EGF-dependent receptor signaling. Based upon these and other observations, the *hypothesis to be tested in this proposal is that LAR plays an important role in EGFR-dependent mammary gland development and tumorigenesis through negative modulation of EGFR signal transduction.*

ANNUAL REPORT

The research question (hypothesis) to be tested in this proposal is that *LAR plays an important role in EGFR-dependent breast cancer through negative modulation of EGFR signal transduction.* With the long term goal of understanding the mechanisms by which EGF signaling is abnormal in breast cancer, two specific aims are being pursued: **First**: characterize the impact of LAR on TGFα-dependent abnormal mammary gland development. This requires a mouse model which has the combined characteristics of a LAR deficient (knockout) mouse and a mouse which has a strong tendency to abnormal mammary development and tumors because of increased expression of the tumor-promoting gene (TGFα) in the mammary gland. The hypothesis would predict that abnormal mammary development will be more pronounced in those mice with the absence of the modulatory effects of LAR. **Second**: define the mechanism by which LAR mediates its modulatory effect on EGFR signaling and alters mammary gland development. Examine how LAR interacts with EGFR. Is it direct or through some intermediate steps?

Aim #1: Characterize the influence of PTP LAR on TGFα-dependent abnormal mammary gland development.

This objective requires the crossing of LAR knockout mouse with mice expressing TGFα targeted to the mammary epithelium. These studies require the monitoring of the mice for tumor formation for a period of 12 months and beyond. In the first funding year, it became known that the co-investigator, Dr. William Kisseberth, was relocating to the Dept. of Clinical Sciences, College of Veterinary Medicine, Ohio State University. While this relocation did not alter the objectives of this study, Dr. Kisseberth's contribution to the project included this aspect of the study, ie. the crossbreeding of the mice and the monitoring of same for tumor formation. Thus, it was decided to postpone the initiation of the mouse crossing studies until after the relocation so that they can be completed at Ohio State. Relocation of the animals during their tumor latency was not considered to be wise. Now that Dr. Kisseberth has been established at Ohio State, he has begun the breeding experiments as described above. We anticipate commencing with the analysis of animal data in the near future. It must be kept in mind that the endpoint is tumor development. Thus, a 12 month delay after the breeding of the mice is needed in order to accumulate the date.

Aim 2: Elucidate the mechanism by which LAR modulates EGFR signaling in the mammary gland.

Two aspects of Aim #2 have shown progress during Year #2. These include **A)** completing final experiments needed to publish our investigation: Expression of PTP LAR is regulated by cell density through functional E-cadherin complexes and **B)** an investigation of the mechanism by which cell contact inhibition (mediated by functional E-cadherin complexes) inhibits EGFR proliferation signaling in a mammary epithelial cell model.

A) Expression of PTP LAR is Regulated by Cell Density Through Functional E-Cadherin Complexes: We have completed this investigation which was desribed in considerable detail in the report for Year 1. Since the major effort in Year 2 on this project was focused on repeating experiments and addressing reviewers' comments before publication, all of the data will not be repeated here. A copy of the preprint is place in the Appendix. Only the Abstract is included below.

Abstract: The receptor-like protein tyrosine phosphatase LAR has been implicated in receptor tyrosine kinase signaling pathways while also displaying cell density dependency and localization to adherens junctions. While physiologic substrates for LAR have not been unequivocally identified, β-catenin associates with LAR and is an *in vitro* substrate. With the implication that LAR may play a role in regulating E-cadherin dependent cell-cell communication and contact inhibition, the relationship of LAR to E-cadherin was investigated.

LAR expression increased with cell density in the human breast cancer cell MCF-7 and in Ln 3 cells derived from the 13672NF rat mammary adenocarcinoma. LAR protein levels rapidly decreased when cells were replated at low density after attaining high expression of LAR at high cell density. COS-7 cells displayed comparable density-dependent regulation of LAR expression when transiently expressing exogenous LAR under the control of a constitutively active promoter, indicating that the regulation of expression is not at the level of gene regulation. Disrupting homophilic E-cadherin complexes by chelating extracellular calcium caused a marked decrease in LAR protein levels. Similarly, blocking E-cadherin interactions with saturating amounts of E-cadherin antibody (HECD-1) also led to a rapid and pronounced loss of cellular LAR. In contrast, mimicking cell surface E-cadherin engagement by plating cells at low density onto dishes coated with HECD-1 resulted in a 2 fold increase in LAR expression compared to controls. These results suggest that density-dependent regulation of LAR expression is mediated by functional E-cadherin and may play a role in density-dependent contact inhibition by regulating tyrosine phosphorylation in E-cadherin complexes.

B) EGFR signaling is inhibited by cell contact at the level of Akt activation: Cell contacts prevent normal adult breast epithelium from proliferating when exposed to growth stimuli, such as EGF. In contrast, neoplastic epithelial cells proliferate in response to EGF despite being surrounded by other cells in the tumor mass. We sought to understand the mechanism of cell contact-mediated inhibition of EGF signaling by comparing EGF-dependent signaling in low density and high density MCF10A cell cultures. The MCF10A normal human breast epithelial cell line requires EGF for proliferation in culture and exhibits density-dependent withdrawal from the cell cycle (fig. 1). EGF treatment caused maximal EGF receptor activation by 10 min that was two-fold higher in the low density MCF10A cells (fig. 2). The EGF receptor remained activated and unchanged for 30 min under both density conditions. EGF receptor mass was also similar in low and high density cultures. Signaling pathways which were independent of cell density include the following: (1) EGF-dependent tyrosine phosphorylation of erbB3 and association of the p85 subunit of PI3 kinase with erbB3 (fig. 3), (2) EGF-dependent tyrosine phosphorylation of Gab-1 (fig. 4), (3) EGF dependent association of the p85 subunit of PI3 kinase with Gab-1 (fig. 4), (4) *In vitro* Gab1-associated PI3 kinase activity (fig. 5), (5) PDK1-dependent phosphorylation of Akt (fig. 5), and, finally (6) Erk-1/2 activation (fig. 6). Only at the level of EGF-dependent Akt activation was a marked difference between high and low density cells apparent (fig. 7). In contact-inhibited MCF10A cells, EGF treatment caused a transient stimulation of Akt activity that peaked at 5 min and rapidly decreased to 30% at 30 min. In the low density cells, in contrast, Akt was activated to the same extent at 5 min as in the high-density cells (as measured by serine 473 phosphorylation), but the low density cells retained 65% of this activity at 30 min (fig. 7). The *in vitro* kinase activity of Akt is higher in the low density cells at both 5 and 30 min (fig. 8). This approximately two fold higher Akt activity in low density cells remains present for at least 21 hours (fig. 9). Preliminary studies show, if low density cells were treated with the PI3 kinase inhibitor LY294002 such that only 30% of the maximal EGF dependent Akt activity was observed at 30 min (i.e. mimicking the high density condition), then EGF no longer stimulated proliferation of these cells (fig. 10). In summary, cell contact mediated suppression of Akt activity blocks proliferation and may identify a critical mechanism for contact inhibition of growth.

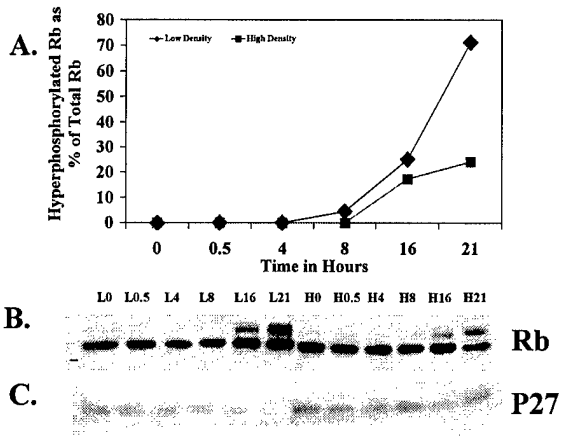

Figure 1. MCF10A cells exhibit density dependent inhibition of growth. MCF10A cultures were grown to confluency (High density). A confluent dish was split 1:4 and reseeded in a larger dish at approximately 15% of its original confluency (Low density). After allowing the cells to attach for 6 hrs., the cells were placed in growth factor free media for 18 hrs. After the Low density, L, and High density, H, were treated for 0, 0.5, 4, 8, 16, or 21 hrs., with 5 ng/ml of EGF, lysates were collected and total cellular protein was determined. Equal amounts of total cellullar protein were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted for **B.** the retinoblastoma protein (Rb), and **C.** P27. **A.** shows the results of a densitometric scan of B. hyperphosphorylated Rb expressed as the percent of total amount of Rb.

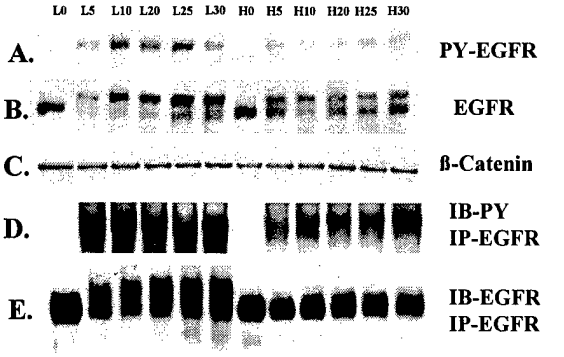

Figure 2. EGF receptor autophosphorylation is greater in low density cells. MCF10A cultures were grown to confluency (High density). A confluent dish was split 1:4 and reseeded in a larger dish at approximately 15% of its original confluency (Low density). After allowing the cells to attach for 6 hrs., the cells were placed in growth factor free media for 18 hrs. After the Low density, L, and High density, H, were treated for 0, 5, 10, 20, 25, or 30 min., with 5 ng/ml of EGF, lysates were collected and total cellular protein determined. Equal amounts of total cellullar protein were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted for **A.** tyrosine-phosphorylated EGF receptor, **B.** EGF receptor mass, and **C.** β-catenin (as a loading control). For **D.** and **E.**, equal amounts of total cellular protein were immunoprecipitated with polyclonal EGF receptor antibodies before separating on SDS-PAGE and immunoblotting with **D.** phosphotyrosine (PY) and **E.** EGF receptor mass.

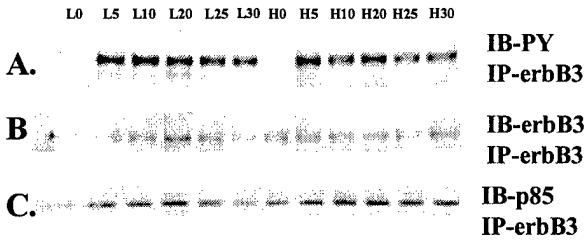

Figure 3. EGF-dependent erbB3 activity is unaffected by density. MCF10A cultures were treated as described in figure 2. After immunoprecipitating with anti-erbB3, the cultures were immunoblotted in **A.** for phosphotyrosine (PY), in **B.** for erbB3 mass, and **C.** for p85 subunit of PI3 kinase.

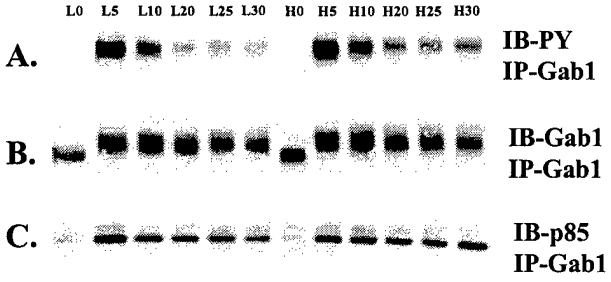

Figure 4. EGF-dependent Gab1 phosphorylation and p85 association of PI3 kinase are unaffected by density. MCF10A cultures were treated as described in figure 3. After immunoprecipitating with anti-Gab1, the cultures were immunoblotted in **A.** for phosphotyrosine (PY), in **B.** Gab1 mass, and **C.** for p85 subunit of PI3 kinase.

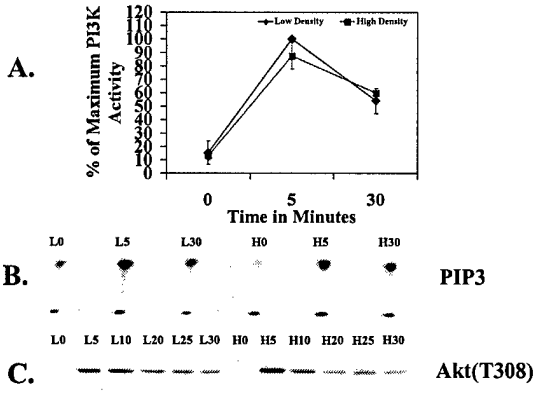

Figure 5. Gab1-associated PI3 kinase and EGF-dependent PDK1 activities are unaffected by density. MCF10A cultures were treated as described in figure 2. After immunoprecipitating with anti-Gab1, PI-3-kinase *in vitro* activity was analyzed, **B. A** shows the average +/- one standard deviation from densitometric scans of 3 experiments. **C.** shows lysates immunoblotted for phospho-Akt(T308) (the site phosphorylated by PDK1). These are representative blots from 3 experiments.

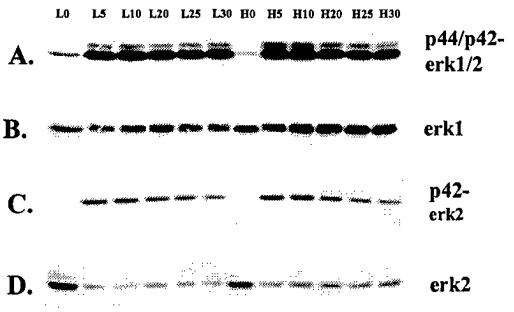

Figure 6. EGF-dependent erk1/2 activity is unaffected by density. MCF10A cultures were treated as described in figure 2. and immunoblotted with **A.** anti- p44/p42 erk1/2, **B.** anti-erk1, **C.** p42 erk2 (lighter exposure), and **D.** anti-erk2. These are representative blots from 3 experiments.

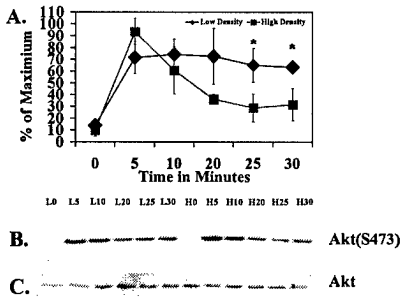

Figure 7. EGF-dependent Akt activity is suppressed by high density. MCF10A cultures were treated as described in figure 2. and immunoblotted with **B.** anti- pSer473 Akt, and **C.** anti-Akt. **A.** Shows the average +/- one standard deviation from densitometric scans of 3 experiments (one shown in B.). * designates student t-test $p < 0.05$.

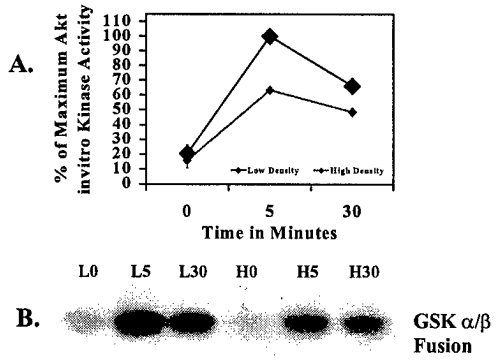

Figure 8. EGF-dependent Akt *in vitro* kinase activity is suppressed by high density. MCF10A cultures were treated as described in figure 2. After immunoprecipitating with anti-Akt, the ability of Akt to phosphorylate glycogen synthase kinase 3 fusion protein (GSK3 Fusion) *in vitro* was measured, **B.** Part **A.** shows the average +/- one standard deviation from densitometric scans of 2 experiments. These results are preliminary pending the result of at least one more experiment.

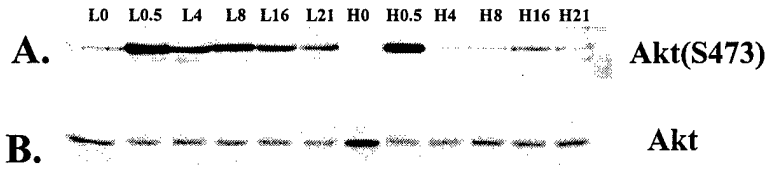

Figure 9. High density cultures suppress EGF-dependent Akt activity for at least 21 hours. MCF10A cultures were treated as described in figure 2. and immunoblotted with **A.** anti- pSer473 Akt, and **B.** anti-Akt.

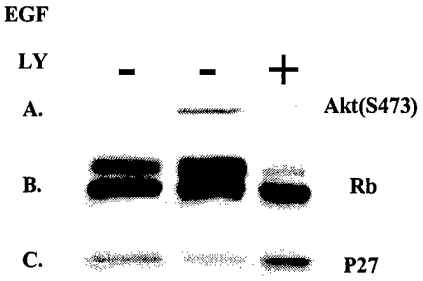

Figure 10. Partial suppression of Akt activity in low density cultures after treatment with the PI3 kinase inhibitor, LY294002, is associated with inhibition of cellular division. Low density cultures were treated for 21 hrs. with or without 5 ng/ml of EGF. After 30 min. of EGF treatment, the cultures were treated with or without 30 μM LY294002. (Prior experiments showed 30 μM LY294002 to inhibit Akt (Ser473) phosphorylation to 30 % of its maximum phosphorylation levels). Lysates were collected and total cellular protein was determined. Equal amounts of total cellular protein were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted for **A.** phospho-Akt (Ser473), **B.** Rb, and **C.** P27.

Conclusions:

1. Although high density cultures have half the EGF receptor autophosphorylation, EGF responses in high and low density cells are comparable down to the level of Akt..
2. Suppression of EGF-dependent Akt activation distinguishes low density from high density cells.
3. When LY294002 is used to artificially suppress Akt activity in low density cultures, cellular division is inhibited.
4. Regulation of EGF-dependent Akt activation may be a critical mechanism for cell contact inhibition of growth.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that expression of the LAR phosphatase in the human breast cancer cell line, MCF7, is dependent upon cell-cell contact.
- Characterized the mechanism by which LAR protein is regulated and demonstrated that functional E-cadherin complexes are necessary and sufficient for this effect.
- This is particularly relevant to the objectives of this project because engagement of E-cadherin complexes are also known to exert an inhibitory effect on EGF receptor signaling, particularly mitogenesis. Might it be that the E-cadherin effect is, in part, mediated through the expression of the PTP LAR as our hypothesis would suggest?
- Cell contact inhibition of EGF-dependent growth is mediated at the level of Akt activation.
- Akt activation if a critical step for control of cell cycle progression, and this inhibition by contact inhibition is present for at least 21 hr after exposure to EGF.

REPORTABLE OUTCOMES

Publication:
Symons, JR, LeVea, C, and Mooney, RA (2002) Expression of PTP LAR is regulated by cell density through functional E-cadherin complexes. *Biochem. J.* 365, 513-519.

Manuscript in preparation:
LeVea, C, and Mooney, RA (2002) Cell contact inhibition of growth is mediated by E-cadherin dependent suppression of EGF-stimulated Akt signaling.

Mouse transgenic model:
Targeted expression of human LAR selectively to the mammary gland via the MMTV promoter.

CONCLUSIONS

Work in the second year has supported the hypothesis that LAR is a negative regulator of EGF receptor signaling. We have characterized the regulation of cellular LAR expression and have revealed an important role for E-cadherin in this process. LAR is regulated by cell density, with concentrations increasing markedly as cell density increases. Functional E-cadherin complexes are necessary for this effect. Tyrosine phosphorylated proteins in the E-cadherin complex may be substrates for LAR. Thus, LAR may regulate E-cadherin complex function. How does this regulate EGF receptor signaling? It is well known that the proliferative effect of EGF receptor signaling is suppressed at confluence, presumably by E-cadherin complexes. We now have shown that EGF proliferative signaling is inhibited at the level of Akt activation. We must now investigate how cell contact inhibition and, presumably E-cadherin/LAR complexes regulate EGFR dependent Akt activity.

Characterization of mammary gland development and tendency to tumorigenesis as a function of LAR expresssion is now underway. These studies will yield important information in the coming months in the laboratory of Dr. Kisseberth at Ohio State.

References: NA

Appendices: 1 preprint

Biochem. J. (2002) 364, 1–7 (Printed in Great Britain) 02/0381

Expression of leucocyte common antigen-related (LAR) tyrosine phosphatase is regulated by cell density through functional E-cadherin complexes

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The receptor-like protein-tyrosine phosphatase leucocyte common antigen-related phosphatase (LAR) has been implicated in receptor tyrosine kinase signalling pathways while also displaying cell-density-dependency and localization to adherens junctions. Whereas physiological substrates for LAR have not been identified unequivocally, β-catenin associates with LAR and is a substrate *in vitro*. With the implication that LAR may play a role in regulating E-cadherin-dependent cell–cell communication and contact inhibition, the relationship of LAR with E-cadherin was investigated. LAR expression increased with cell density in the human breast cancer cell line MCF-7 and in Ln 3 cells derived from the 13762NF rat mammary adenocarcinoma. LAR protein levels decreased rapidly when cells were replated at a low density after attaining high expression of LAR at high cell density. COS-7 cells displayed comparable density-dependent regulation of LAR expression when transiently expressing exogenous LAR under the control of a constitutively active promoter, indicating that the regulation of expression is not at the level of gene regulation. Disrupting homophilic E-cadherin complexes by chelating extracellular calcium caused a marked decrease in LAR protein levels. Similarly, blocking E-cadherin interactions with saturating amounts of E-cadherin antibody (HECD-1) also led to a rapid and pronounced loss of cellular LAR. In contrast, mimicking cell-surface E-cadherin engagement by plating cells at low density on to dishes coated with HECD-1 resulted in a 2-fold increase in LAR expression compared with controls. These results suggest that density-dependent regulation of LAR expression is mediated by functional E-cadherin and may play a role in density-dependent contact inhibition by regulating tyrosine phosphorylation in E-cadherin complexes.

Key words: β-catenin, MCF-7.

INTRODUCTION

E-cadherin is a member of a family of calcium-dependent transmembrane proteins which play a central role in cell–cell communication and contact inhibition [1,2]. These functions are mediated by homophilic binding between extracellular domains of E-cadherin expressed on neighbouring cells and localized to adherens junctions. The intracellular domain of E-cadherin contains binding sites for several proteins, including β-catenin, γ-catenin (plakoglobin) and p120 catenin. Tight cell–cell adhesion is achieved through these protein–protein interactions, which form a bridge to the actin filaments. The E-cadherin intracellular domain binds β-catenin, which in turn binds α-catenin. It is thought that α-catenin interacts directly or indirectly (via α-actinin) with the F-actin bundles, linking the complex to the actin cytoskeleton and forming a stable structure when laterally clustered [3].

Loss of E-cadherin in transformed epithelial cells has been correlated with increased tumour invasiveness *in vitro* and with tumour progression *in vivo* [4,5]. In breast cancer, loss of functional E-cadherin complexes correlates with a loss of the epithelial phenotype and increased invasiveness [5]. E-cadherin and the proteins within the cadherin complex are thought to be post-translationally modified to provide a mechanism for rapid turnover and modulation of the functional state. Several of these proteins, including p120 catenin, E-cadherin, γ-catenin (plakoglobin) and β-catenin, are apparently regulated by tyrosine phosphorylation [7–9]. Whereas tyrosine phosphorylation of β-catenin appears not to promote its uncoupling from cell adhesion complexes directly [10], it may be involved in a mechanism for disruption of functional E-cadherin complexes. Studies *in vitro* have identified several protein tyrosine phosphatases (PTPs) that bind to and alter the phosphorylation state of β-catenin. Among the kinases, the epidermal growth factor (EGF) receptor and erb-B2 associate with β-catenin *in vitro* and *in vivo* [11,12]. A β-catenin mutant lacking its N-terminal half, including much of the central armadillo motifs, cannot bind E-cadherin but binds to erb-B2. This mutant inhibits the tyrosine phosphorylation of endogenous β-catenin associated with the E-cadherin complex [12]. Cells transfected with this deletion mutant display decreased transforming growth factor-α-dependent migration *in vitro* and decreased metastasis *in vivo*. The hepatocyte growth factor (HGF) receptor/c-Met receptor has been shown to co-localize and associate with E-cadherin and β-catenin in epithelial tumour cells [13]. Following c-Met activation with HGF/~~stem~~ factor, tyrosine phosphorylation of β-catenin occurs and cell–cell contacts are disrupted [14]. The Src kinase has been implicated in this HGF-dependent process [15]. Rat fibroblasts become metastatic and lose their compact aggregating morphology when transformed with v-Src [16]. Tyrosine kinase inhibitors restore tight cell–cell adhesion. β-Catenin is the most significantly increased tyrosine-phosphorylated protein of the cadherin-associated proteins in the presence of v-Src [16]. Despite these observations, tyrosine phosphorylation of β-catenin as a mechanism for disruption of functional E-cadherin complexes remains controversial [10].

Several PTPs have also been shown to associate with cadherin complexes [17–21]. PTP activity appears to be necessary for strong cell–cell adhesion since cell–cell contact can be disrupted by treatment with the PTP inhibitor pervanadate [22]. Studies *in vitro* have demonstrated that leucocyte common antigen-related

Abbreviations used: EGF, epidermal growth factor; HGF, hepatocyte growth factor; LAR, leucocyte common antigen-related phosphatase; PTP, protein tyrosine phosphatase; E-subunit, extracellular subunit; P-subunit, PTP-domain-containing subunit.

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phosphatase (LAR) associates with the N-terminus of β catenin and the closely related γ-catenin [17]. β-Catenin has also been shown to be a substrate for LAR *in vitro* [23]. These data suggest that LAR may play a role in the maintenance of cell–cell contacts by regulating the tyrosine-phosphorylation state of one or more proteins in the E-cadherin complex. Here, we expand upon the earlier report that LAR is regulated by cell density [24]. Functional E-cadherin complexes are shown to regulate expression of LAR through a mechanism that does not require changes in rates of transcription. Regulation of LAR expression may be a mechanism by which E-cadherin complexes control their own functional state.

EXPERIMENTAL

Cell lines

COS-7 green monkey kidney cells were obtained from the A.T.C.C. Ln 3 rat mammary adenocarcinoma cells were a generous gift from Dr Carl McCary (University of Rochester, Rochester, NY, U.S.A.). MCF-7 cells were provided by Dr P. J. Simpson-Haidaris (University of Rochester).

Plasmids and transfections

The mouse E-cadherin construct was a generous gift from Masatoshi Takeichi of Kyoto University, Kyoto, Japan, and has been described previously [25]. Human LAR cDNA (kindly provided by Michel Streuli of the Dana Farber Cancer Institute, Boston, MA, U.S.A.) was cloned into the pcDNA3 vector in frame with a 5′ Myc/His tag. Bacterial transformation was performed using subcloning efficiency DH5α from Life Technologies. Plasmid preparations were performed using Qiagen maxiprep kits according to their protocol. DNA concentration and purity were measured by UV spectroscopy. Transient transfections of cell lines were performed using the Fugene 6 reagent (Roche) under serum-free conditions as described by the manufacturer. Transfected cells were maintained for 36 h before experimentation.

Antibodies and reagents

Mouse monoclonal antibodies specific for β-catenin, E-cadherin and the LAR E-subunit were from Transduction Laboratories. The anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology. Antibody for the LAR P-subunit (PTP domain containing subunit) was provided by Dr Barry Goldstein of Thomas Jefferson University (Philadelphia, PA, U.S.A.). Antibodies to human E-cadherin (HECD-1) and rat E-cadherin (DECMA-1) were from Zymed. All other reagents were purchased from Sigma unless otherwise indicated.

Immunoprecipitations and Western blotting

When direct analysis of cell lysates was appropriate, cells were scraped into Laemmli's lysis buffer (156 mM Tris/HCl, 5% SDS and 2.5% glycerol) at 100 °C. After normalizing samples based on protein using the Markwell–Lowry protein assay, proteins were separated by SDS/PAGE.

When immunoprecipitation of proteins was required, cells were scraped into lysis buffer [1 M Tris/HCl, pH 7.4, 2 M NaCl, 1 M NaF, 10% Triton X-100, 200 mM PMSF, benzamidine, tetrasodium pyrophosphate, *p*-nitrophenylphosphate, pervanadate and a protease-inhibitor cocktail (Calbiochem)]. Lysates were passed 10 times through an 18-gauge needle. Samples were protein-normalized using the Bradford assay. The immunoprecipitating antibody was pre-bound to Protein G–Sepharose beads (Amersham Bioscience) before lysates were added at 4 °C for 1–4 h. Beads were washed three times with Triton wash buffer (1% Triton X-100, 100 mM Tris/HCl and 150 mM NaCl) before immunoprecipitates were eluted with boiling Laemmli sample buffer (78 mM Tris/HCl, 2.5% SDS, 2.5% glycerol and 2 mM dithiothreitol). Samples were transferred to either PVDF or nitrocellulose membranes and analysed by Western blotting. The blots were developed with either Amersham Bioscience or Pierce chemiluminescence reagents.

Cell-density-dependent plating assays

Following propagation to confluence, cells were rinsed, dispersed with 0.25% trypsin for 1–5 min and resuspended in standard serum-containing media. Cells were pooled and concentrated by centrifugation. Aliquots were re-plated at high (> 85%) or low (< 30%)density in 100 mm dishes. At appropriate time points, cells were lysed and protein expression analysed by Western blotting as described above.

Disruption of E-cadherin complexes

MCF-7 cells were grown to confluence in 24 well dishes. Cell cell contact was disrupted with either EGTA or HECD-1, a mouse monoclonal anti-E-cadherin antibody. For EGTA treatment, EGTA was added at 5 mM with a media change followed by incubations for 6 h or less. Incubations of 18 h and longer were found to be cytotoxic. To block E-cadherin more specifically, MCF-7 cells were incubated in media containing the E-cadherin-blocking antibody, HECD-1, at 1, 5 or 10 μg/ml for 18–24 h. Cells were examined by light microscopy at several time points to ensure cell viability and to record morphological changes induced by treatment. Protein expression was analysed as described above.

RESULTS

LAR protein levels change with cell density

Our previous studies indicated that LAR protein expression is cell-density-dependent [24]. In light of the observed association of LAR with constituents of the adherens junctions [17,23,26] and the defect in terminal differentiation of the mammary gland in LAR-knockout mice [27], the current study was initiated to explore the mechanism of cell-density-dependency of LAR using breast cancer cell lines. LAR protein levels were examined in the human MCF-7 breast carcinoma cell line and in a rat mammary adenocarcinoma cell line, Ln 3. Each cell type was grown for several passages at either high (85–100% confluence) or low (10–30% confluence) cell density. The MCF-7 cells were additionally examined at 40–60% confluence (medium density). Cell lysates were normalized for protein and separated on SDS/PAGE gels. In the human MCF-7 cell line, LAR protein increased markedly from low to high density as detected by Western blotting using an antibody to the LAR E-subunit (Figure 1A). It was possible that the difference in LAR E-subunit expression did not reflect expression of the catalytically active P-subunit. For example, the E-subunit alone may have been lost at low cell density due to proteolytic cleavage or shedding of this extracellular domain [26]. However, when MCF-7 lysates were probed with an antibody to the catalytic or P-subunit of LAR, comparable density-dependent changes in LAR expression were observed. Similarly, the highly metastatic Ln 3 cells, derived from the 13762NF rat mammary adenocarcinoma [28], exhibited a large increase in LAR protein from low to high density when detected with the P-subunit antibody (Figure 1B). The less metastatic Ln 2 cells, also derived from the 13762NF tumour,

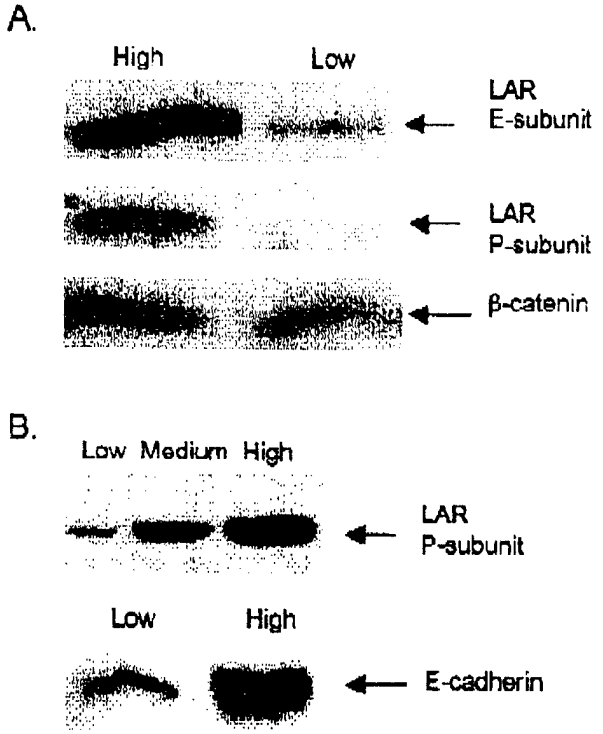

Figure 1 Effect of cell density on LAR and E-cadherin expression

MCF-7 (**A**) and Ln 3 (**B**) cell lines were grown for several passages at high (> 65%), medium (approx. 40–60%), or low (< 30%) confluence. After cell lysis, proteins were separated by SDS/PAGE and transferred to nitrocellulose. LAR subunit expression in MCF-7 cells (**A**) and expression of LAR and E-cadherin in Ln 3 cells (**B**) were detected by Western blotting as described in the Experimental section. β-Catenin was used as a loading control in (**A**).

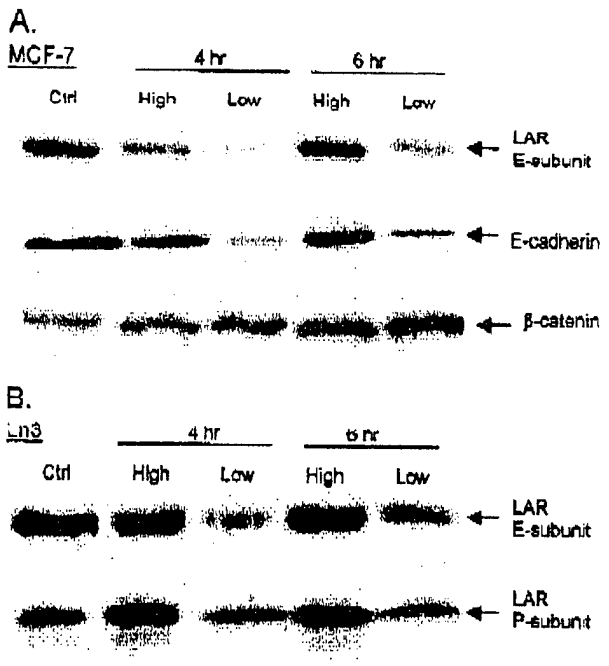

Figure 2 Rapid decrease in LAR expression with a decrease in cell density

MCF-7 (**A**) and Ln 3 (**B**) cells, which had been maintained at confluence, were resuspended with trypsin and replated at either high (> 65%) or low (< 30%) confluence for either 4 or 6 h. Changes in expression of LAR E-subunit (**A**, **B**), LAR P-subunit (**B**), E-cadherin (**A**) and β-catenin (**A**) were determined by Western blotting. Ctrl, control.

showed a progressive increase in LAR levels from low to high density similar to the Ln 3 cell line (results not shown). Analysis of E-cadherin levels as a function of cell density paralleled LAR levels in Ln 3 cells.

LAR levels respond to rapid changes in cell density

The following investigation examined the impact of acute changes in cell density on LAR expression. Cells were grown to confluence before being dissociated, pooled and replated at either high or low density. Within 4 h of replating, LAR protein levels decreased markedly when replated at low density. In contrast, replating at high density preserved the pre-existing LAR expression (Figure 2). E-cadherin levels decreased in parallel with those of LAR when cells were exposed to low-cell-density conditions. In contrast, β-catenin, which was used as a loading control, showed no density-dependent changes, though a small increase was observable as a function of time after replating. The decreased expression of LAR reflected changes in both the E- and P-subunits. Whereas the E-subunit potentially could have been influenced by shedding, the similar density-dependent changes in LAR observed in Ln 3 cells (Figure 2B) probed with either a P-subunit or E-subunit antibody argue that decreased cell density initiated the loss of total cellular LAR protein.

The above investigation demonstrated that LAR levels decreased markedly within 4 h of an acute decrease in cell density. Next the response to an acute increase in cell density was investigated. Cells were maintained at 10–30% confluence for several days before harvesting and replating at high density. LAR protein expression was analysed by Western blotting at subsequent times as indicated in Figure 3. LAR was modestly increased within 8 h of replating at high density and continued to increase more substantially from 8 to 24 h. By 24 h, the level of LAR protein reached that of the high-density control cells (results not shown). Because MCF-7 cells do not proliferate rapidly and the cells had been plated at high density, the increase in LAR protein expression was not likely to be due to cell proliferation. The more modest increase in β-catenin and the

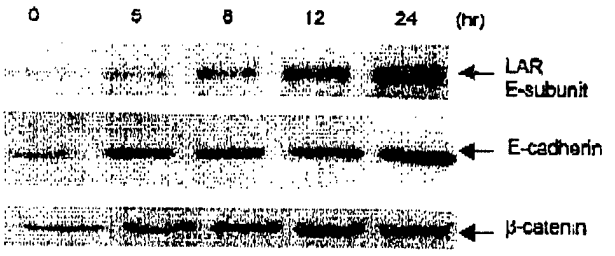

Figure 3 Slow increase in LAR expression with a rapid increase in cell density

MCF-7 cells were maintained at low density (< 30%) for several passages. Following resuspension with trypsin, pooled cells were replated at high cell density. After replating, LAR, E-cadherin and β-catenin expression were determined by Western blotting at the indicated time points.

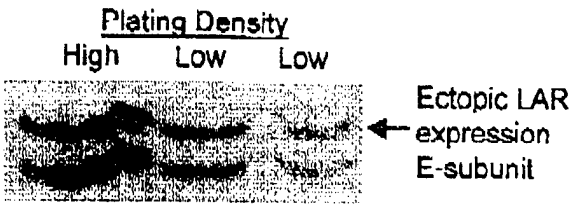

Figure 4 Density-dependent regulation of ectopically expressed LAR

COS-7 cells were transiently transfected with a human LAR cDNA construct as described in the Experimental section. At 36 h post-transfection, cells were resuspended with trypsin, pooled and replated at either high (> 85%) or low (< 30%) confluence for 6 h. Experiments with the low-confluence condition were performed in duplicate. LAR expression was determined by Western blotting.

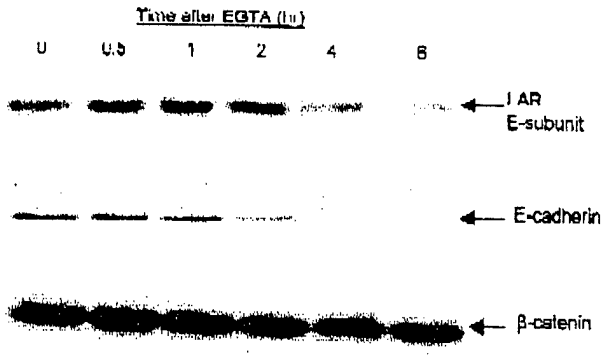

Figure 5 Effect of EGTA-dependent disruption of cell–cell contacts on expression of LAR and E-cadherin

Confluent MCF-7 cells were treated with 5 mM EGTA for the time periods indicated and then harvested. LAR, E-cadherin and β-catenin (loading control) expression were determined in the cell lysates using Western blotting.

absence of an increase over the time period (12–24 h) that corresponds to the large increases in LAR and E-cadherin supports this conclusion. Interestingly, the most substantial increases in LAR protein expression occurred subsequent to the formation of cell–cell contacts. As observed by light microscopy, the MCF-7 cells began to spread at 4–6 h and visible cell–cell contacts were observable at 8–10 h. Whereas LAR levels increased substantially after 8 h, maximum levels were not reached for an additional 16 h. Thus the slow rate of increase in LAR mass with an acute increase in cell density is in marked contrast to the very rapid loss of LAR with acute decrease in cell density. It is logical to speculate that *de novo* synthesis of LAR, and perhaps other proteins, is required to mediate the observed changes. Finally, increases in LAR protein levels paralleled those of E-cadherin in response to the increase in cell density. From the above data, we hypothesize that LAR protein levels increase with the formation of functional cell–cell contacts.

Density-dependent changes in LAR levels do not require changes in the synthetic rate

To address the possibility that the difference in LAR levels at varying densities was due to differing rates of transcription, we used a method similar to that of Gebbink et al. [29]. COS-7 cells were transiently transfected with LAR cDNA under the control of a constitutively active promoter. The transfected cells were incubated for 24 h, dissociated and replated at either high (> 85%) or low (< 30%) density. The cells were lysed 24 h later and LAR expression was analysed. Since exogenous LAR expression is under the control of a constitutively active promoter, the transcription rate of LAR remains constant whether cells are at high or low densities. Changes in LAR levels would be due to altered rates of protein degradation or mRNA stability and translation. As shown in Figure 4, LAR expression in transfected COS cells, which express no detectable endogenous LAR, increased several-fold with increasing cell density. Thus regulation of LAR transcription rate is not responsible for density-dependent LAR expression. Ectopically expressed LAR in COS cells appeared as a doublet by Western blotting. One potential explanation is that these bands represent alternatively spliced isoforms of the extracellular domain of LAR. Zhang et al. [30] have described a 27 bp exon encoding a nine amino acid sequence in the E-subunit of LAR that is alternatively spliced. This group has shown that expression of these isoforms is expressed in a developmentally regulated pattern in the brain. While the co-expression of two LAR isoforms is not observed routinely in transfection experiments, doublets of the E-subunit have been observed by Western blotting following transfection [31,32]. An alternative explanation for the presence of a doublet in LAR Western blots is that one or both represent *in vitro* proteolytic fragments. The ratio of the two immunoreactive bands remained relatively constant at approx. 1.0 among samples and between experiments. This and the absence of a doublet with endogenous LAR argue for a cell-dependent processing rather than an *in vitro* artifact, such as proteolysis.

LAR protein levels are altered by disruption of E-cadherin-mediated cell–cell contact

We hypothesized that cell-density-dependent increases in LAR protein expression were due to a signal mediated by E-cadherin-dependent cell cell contact. To pursue this possibility, MCF-7 cells were treated with EGTA to disrupt cell–cell contact through chelation of the extracellular calcium required for E-cadherin homophilic binding between cells. Following EGTA treatment, the cells were examined by light microscopy to document morphological changes. By 1 h of treatment with EGTA, MCF-7 cells became rounded and detached from one another. At 6 h, MCF-7 cells had no cell–cell contacts, but the cells remained attached to the culture dishes. By 18 h, cells had begun to lift from the dish and marked cell death was apparent. Cells were lysed at these various time points (excluding 18 h) and protein-normalized lysates were separated on SDS/PAGE gels. Western blotting for the LAR E-subunit revealed that LAR was expressed in high amounts at 0, 1 and 2 h, but decreased sharply after 2 h (Figure 5). After 6 h LAR protein was undetectable (results not shown). E-cadherin protein levels were also examined to determine whether loss of cell–cell contact induced by EGTA also caused changes in E-cadherin expression. E-cadherin protein was also high at 0 and 1 h, but began to decrease by 2 h (Figure 5). As a control for protein loading, cell lysates were probed for β-catenin. There was no change in β-catenin protein expression at any time point (Figure 5). Whereas both E-cadherin and LAR levels decreased with EGTA treatment, the decrease in E cadherin commenced at an earlier time point than that of LAR. This indicates that total cellular E-cadherin does not closely correlate with LAR under these conditions. LAR did, however, decrease in response to disruption of cell–cell contacts. What is not assessed here is the impact of calcium chelation on the cellular clearance mechanisms for E-cadherin and LAR. It has been demonstrated, for example, that calcium mobilization with

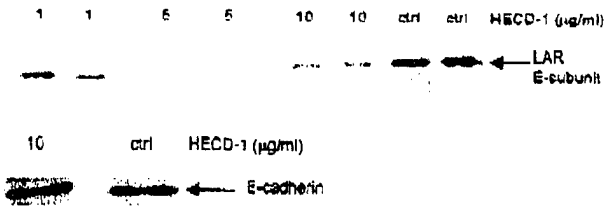

Figure 6 Effect of E-cadherin-blocking antibody on expression of LAR and E-cadherin

Confluent MCF-7 cells were treated for 24 h with media containing the E-cadherin-blocking antibody HECD-1 at the concentrations indicated (μg/ml) or the IgG control (ctrl). Cells were harvested at 24 h and LAR and E-cadherin expression were determined in cell lysates using Western blotting.

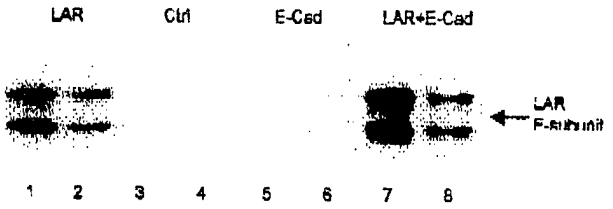

Figure 7 Effect of ectopic E-cadherin expression on density-dependent regulation of LAR

COS-7 cells were transiently transfected with constructs for LAR, E-cadherin (E-Cad) or null vector (Ctrl) as indicated. After 48 h, cells were resuspended and replated at high (lanes 1, 3, 5 and 7) or low density (lanes 2, 4, 6 and 8) for 6 h. LAR expression was determined using Western blotting.

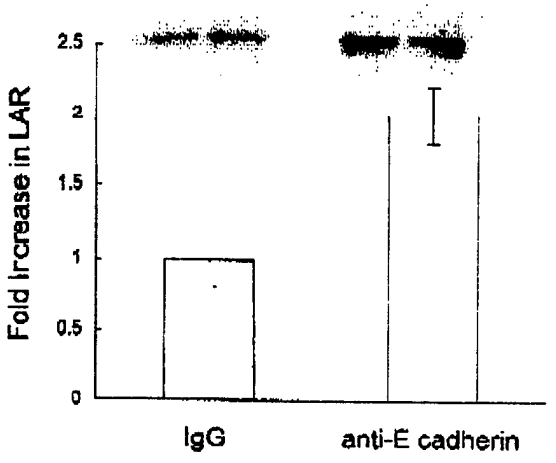

Figure 8 E-cadherin-dependent increase in LAR expression

MCF-7 cells were plated at low density on to culture dishes coated with either anti-E-cadherin antibody (HECD-1) or rabbit IgG as a control. Cells had been maintained at low density for the previous 48 h. After 12 h, cells were harvested and LAR expression determined by Western blotting. Quantitative results represent the means ± S.D. from three experiments each performed in duplicate.

ionophore A23187 induces proteolysis of cellular LAR [26]. EGTA blocks this ionophore-dependent proteolytic process. It is possible that chelation of calcium with EGTA in our experiments retarded the clearance of LAR that was otherwise initiated by the disruption and clearance of E-cadherin.

Since expression levels of LAR and E-cadherin were observed to change in parallel under most, though not all, conditions, we sought to determine whether a more direct relationship between the two proteins could be established. The homophilic cadherin-mediated cell–cell interactions are the molecular sensors of cell density. Thus we hypothesized that functional E-cadherin interactions, not necessarily total cellular E-cadherin content, may be responsible for the density-dependent change in LAR protein levels. To determine whether functional cadherin complexes control density-dependent LAR expression, we inhibited extracellular cadherin interactions directly by using an E-cadherin-specific monoclonal blocking antibody, HECD-1. Confluent MCF-7 cells were treated with 1, 5 or 10 μg/ml of HECD-1 to inhibit cell–cell contact. When the cells were analysed for morphological changes, light microscopy revealed that 5 and 10 μg/ml HECD-1 caused greater than half of the cells to round up (results not shown). When 1 μg/ml antibody was used, the cells maintained their cell cell adhesive properties. Western blotting showed a marked decrease in LAR expression when HECD-1 was added at 5 or 10 μg/ml as compared with the control (Figure 6). Thus cadherin complexes mediate the expression of LAR protein. Under the same conditions, E-cadherin protein levels were not reduced, indicating that functional E-cadherin interactions rather than just cellular E-cadherin expression regulated LAR expression.

E-cadherin alone is not sufficient for the regulation of LAR

Since inhibition of E-cadherin homophilic interactions resulted in decreased LAR expression, we hypothesized that functional E-cadherin complexes were responsible for density-dependent regulation of LAR expression, perhaps by sequestering LAR at adherens junctions and decreasing its turnover. To examine this possibility, COS-7 cells were transiently co-transfected with mouse E-cadherin and human LAR. Transfection efficiency of E-cadherin was approx. 30–40 % as assessed by immunocytochemistry. Whereas we did not have an appropriate antibody to determine the transfection efficiency of LAR, Western blotting revealed cellular expression levels of LAR that exceeded those of primary hepatocytes and several hepatoma cell lines, including HepG2 cells. It was assumed that the transfection efficiency of E-cadherin was sufficient to influence the cellular level of transfected LAR if such an interaction was occurring. While the results were variable and the data in Figure 7 show some increase in LAR levels with co-expression of E-cadherin, it was concluded that exogenous E-cadherin did not consistently alter the exogenous expression of LAR in these cells (Figure 7). Nevertheless, protein levels of exogenously expressed LAR continued to be regulated by cell density in the co-transfected COS-7 cells (see also Figure 4), suggesting that E-cadherin alone is not sufficient to regulate LAR expression.

Activation of E-cadherin complexes is sufficient to increase LAR expression

Functional E cadherin complexes appear to be involved in the regulation of LAR expression. Perhaps ectopic expression of E-cadherin did not increase LAR levels because it did not appreciably increase the amount of functional E-cadherin complexes. To more directly investigate the role of functional E-cadherin complexes in the regulation of LAR levels, MCF-7 cells that had been maintained at low density for a minimum of 48 h were replated on culture dishes coated with either the E-cadherin antibody HECD-1 or rabbit IgG. The work of Lambert et al. [33]

has shown that interactions of cells with anti-cadherin-antibody-coated surfaces will mimic cadherin-mediated cell contact formation, recruitment of β-catenin, α-catenin and p120, and initiate cytoskeleton reorganization. Here, cells were plated at low density with little opportunity for cell cell contact. Relative to LAR expression in control cells, LAR increased 2-fold in cells plated on the anti-E-cadherin antibody (Figure 8). Cell proliferation did not differ between the two sets of conditions during the course of the incubation.

DISCUSSION

In this study, LAR protein expression was shown to be responsive to cell density in mammary carcinoma cell lines, and functional E-cadherin complexes appeared to be the essential mediators or density sensors for this process. Additionally, transcriptional regulation did not appear to play a role in the changes in LAR expression. While the current study demonstrates that LAR levels are under the control of the E-cadherin complex, it has also been shown that LAR levels are sensitive to calcium ionophore and phorbol ester treatments [26]. Under the influence of these agents, LAR and the closely related PTPσ were shown to undergo proteolytic cleavage and subsequent shedding of their E-subunit. Loss of the E-subunit was followed by internalization of the catalytic P-subunit as shown by immunohistochemistry. The ultimate fate of the P-subunit was not determined, though P-subunit remained in the cytosol 4 h after treatment. The E-cadherin-dependent changes in LAR levels that are described in the current study with MCF-7 and Ln 3 cells indicate that comparable proportions of the P- and E-subunits are lost from cells in 4 h with a decrease in cell–cell contact. Although it cannot be ruled out that the E-subunit is being shed prior to the loss of the P-subunit, the ultimate result of the loss of cell–cell contact via decreased E-cadherin homophilic interactions is a loss of both subunits of the LAR protein. Using conditions that were similar to those used in the present study (i.e. disruption of cell–cell adhesion), Aicher et al. [26] showed that EGTA treatment of A431 cells resulted in internalization of β-catenin, plakoglobin and LAR. In this case, the entire LAR molecule was internalized (i.e. E- and P-subunits). While this internalization is assumed to lead to degradation, no comparison of the rates of loss of cellular LAR and E-cadherin were performed. Nonetheless, this report supports our conclusion that disruption of cell–cell adhesion results in removal of LAR and supports a mechanism by which the entire LAR molecule is internalized and degraded in response to a decrease in functional E-cadherin complexes.

LAR is one of several PTPs whose expression levels are influenced by cell density. We reported previously that levels of LAR, PTP1B and SHP2 (SH2-domain-containing PTP2) increase with cell density in the rat McArdle hepatoma cell line, RH7777 [24]. PTP DEP-1 (high-cell-density-enhanced PTP1) was first characterized by its increased expression and activity in high-density cultures [34]. PTPμ has been shown to be up-regulated with increasing cell density through homophilic binding between its extracellular domains expressed on adjacent cells [29]. This is not dissimilar from the mode of interaction of the cadherin family of transmembrane proteins. It has been hypothesized that PTPμ is up-regulated at high density because its homophilic interactions stabilize the PTP at the cell surface [29] and prolong its half-life. In contrast to this mechanism for stabilization of PTPs at high cell density, there is no evidence that the extracellular domain of LAR exhibits homophilic binding capability. Thus there is likely to be a mechanism other than homophilic interactions for density-dependent regulation of LAR. One report indicates, however, that one splice variant of LAR can bind to laminin–nidogen complexes via its extracellular domains [35]. This has not, however, been shown to affect the half-life of LAR in cells. Finally, our studies indicate that regulation of transcription is not an essential step in E-cadherin-mediated regulation of LAR expression. Density-dependent LAR expression is observed even when ectopic LAR expression is driven by a constitutive promoter.

Based on published data [17,23,26] and our current observations, we support a model in which LAR is sequestered in functional E-cadherin complexes by an association that stabilizes LAR and prolongs its half-life. In the absence of or in response to disruption of these E-cadherin-dependent associations, LAR is subject to a rapid turnover, as we have observed when cells are moved to low-cell-density conditions after being maintained for several days under conditions of high cell–cell contact (i.e. high density). However, our data suggest that LAR does not associate directly with E-cadherin. When both LAR and E-cadherin were ectopically expressed in COS cells, E-cadherin expression had little or no effect on the level of LAR expression. The ectopic expression of E-cadherin may not form additional functional complexes with which to sequester additional LAR. Nonetheless, the inability of E-cadherin to affect LAR levels when both are ectopically expressed in COS at low cell density indicates that E-cadherin alone is insufficient to modulate LAR levels and suggests that other components of a functional E-cadherin complex are required for the density-dependent regulation of LAR.

Whereas E-cadherin expression alone is not sufficient for modulating density-dependent increases in LAR expression, E-cadherin is necessary for this process in MCF-7 cells. This was demonstrated with cells that were plated on to a surface coated with the HECD-1 antibody. Interactions between anti-cadherin antibodies and cell-surface cadherin have been shown to mimic cell-contact formation, cadherin clustering, recruitment of the components of the activated cadherin complex and cytoskeleton rearrangement [33]. In our investigations, such antibody-mediated activation was accompanied by a 2-fold increase in LAR expression. These data demonstrate that the density-dependent increase in LAR is related directly to functional E-cadherin interactions.

Whereas LAR does not appear to associate directly with E-cadherin, evidence both *in vivo* and *in vitro* supports a direct interaction between LAR and β-catenin [17,23]. Since β-catenin is an integral component in the E-cadherin complex, along with α-catenin, plakoglobin (γ-catenin) and p120 catenin, its physical association with LAR may explain the correlation of LAR expression with functional E-cadherin complexes. Kypta et al. [17] first reported the association of LAR with β-catenin in PC12 cells and indicated that the N terminal domain of β catenin is necessary for this association. Neither the armadillo domains of β-catenin nor association with α-catenin are necessary for an association *in vitro*. Muller et al. [23] have shown more recently that β-catenin is a substrate for LAR *in vitro*. They observed that ectopic expression of LAR inhibits epithelial cell migration induced by EGF. This was associated with a decrease in the free, uncomplexed pool of β-catenin and in the tyrosine phosphorylation of this population of β-catenin. It has been suggested by several groups [16,36,37] that tyrosine phosphorylation of β-catenin may disrupt the interaction of the E-cadherin complex with the actin cytoskeleton. While this remains controversial, increased free β-catenin may indicate disruption of E-cadherin complexes. LAR may regulate this process, which is important to cell migration by controlling phosphorylation of β-catenin.

The data in the current study now add to this model by demonstrating that cellular levels of LAR are controlled by the

functional E-cadherin complex. Whereas stoichiometric studies were not possible, it is interesting to speculate that cellular LAR levels reflect the number of functional E-cadherin complexes. More specifically, these LAR levels may reflect β-catenin sequestration in the complexes. Muller et al. [23] have shown that free, uncomplexed β-catenin increases with EGF treatment. If this pool of free β-catenin is derived from E-cadherin complexes, might this altered localization affect the associated LAR molecule? In preliminary studies, we have observed that LAR levels decrease in response to EGF in a time course that is consistent with increases in free β-catenin (C. M. LeVea and R. A. Mooney, unpublished work).

In summary, tyrosine phosphorylation in E-cadherin complexes may be an essential regulatory mechanism to maintain cell–cell contact or to control migration in response to extracellular stimuli. Here we demonstrate that cellular levels of the transmembrane PTP LAR are regulated by functional E-cadherin complexes. The increased LAR levels at high cell density may represent a cellular mechanism to suppress further tyrosine phosphorylation of β-catenin (and plakoglobin?) in the E-cadherin complex and maintain contact inhibition of growth.

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